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Alterations in the Hydrophilic Segment of the Maltose-Binding Protein (MBP) Signal Peptide That Affect Either Export or Translation of MBP†

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Mutations that reduce the net positive charge within the hydrophilic segments of the signal peptides of several prokaryotic exported proteins can result in a reduction in the rate of protein export, as well as a reduction in protein synthesis (M. N. Hall, J. Gabay, and M. Shwartz, EMBO J. 2:15–19, 1983; S. Inouye, X. Soberon, T. Franceschini, K. Nakamura, K. Itakura, and M. Inouye, Proc. Natl. Acad. Sci. USA 79:3438–3441, 1982; J. W. Puziss, J. D. Fikes, and P. J. Bassford, Jr., J. Bacteriol. 171:2302–2311, 1989). This result has been interpreted as evidence that the hydrophilic segment is part of a mechanism that obligatorily couples translation to protein export. We have investigated the role of the hydrophilic segment of the *Escherichia coli* maltose-binding protein (MBP) signal peptide in the export and synthesis of MBP. Deletion of the entire hydrophilic segment from the MBP signal peptide resulted in a defect in MBP export, as well as a dramatic reduction in total MBP synthesis. Suppressor mutations that lie upstream of the *malE* coding region were isolated. These mutations do not affect MBP export but instead were shown to partially restore MBP synthesis by increasing the efficiency of MBP translational initiation. In addition, analysis of a series of substitution mutations in the second codon of certain *malE* alleles demonstrated that MBP export and synthesis can be independently affected by mutations in the hydrophilic segment. Finally, analysis of alterations in the hydrophilic segment of the ribose-binding protein signal peptide fused to the mature moiety of the MBP has revealed that the role of the hydrophilic segment in the export process can be functionally separated from any role in translation. Taken together, these results strongly suggest that the hydrophilic segment of the MBP signal peptide is not involved in a mechanism that couples MBP translation to export and argue against the presence of a mechanism that obligatorily couples translation to protein export in *Escherichia coli*.

Proteins destined for export in *Escherichia coli* are synthesized as precursor species with an amino-terminal extension termed the signal peptide that is responsible for initiating the export of proteins from the cytoplasm. The periplasmic maltose-binding protein (MBP) of *E. coli*, the product of the *malE* gene, is synthesized with a typical bacterial signal peptide that shares little primary amino acid homology with other signal peptides but does exhibit conserved features characteristic of signal peptides of both prokaryotic and eukaryotic origin. The first eight residues of the MBP signal peptide constitute the hydrophilic segment, a distinct region that carries a net positive charge due to the presence of three basic residues. The hydrophilic segment precedes the hydrophobic core, a region devoid of charged residues that is predicted to assume an α-helical conformation. Following the hydrophobic core is the cleavage site for signal peptidase I. (For a comprehensive review of the MBP signal peptide and MBP export, see reference 4.)

A specific role for the basic residues of the hydrophilic segment in protein export was first suggested in the loop model (18). The basic residues were proposed to initiate an ionic interaction between the signal peptide and the negatively charged inner face of the cytoplasmic membrane. More-recent studies have provided support for a model in which basic residues either adjacent to the transmembrane domains of integral membrane proteins or in the signal peptides of secreted proteins act as topological determinants of transmembrane orientation (20, 25, 26, 43, 44; for a review, see reference 5). It has also been suggested that the hydrophilic segment may play a role in interactions with components of the cellular protein export machinery. Chemical cross-linking studies have indicated that the basic residues of the hydrophilic segment may specifically interact with the SecA protein (1). In addition, Puziss et al. (25) reported that *prlD2*, an allele of the *secA* gene, was capable of suppressing the export defect associated with alterations in the hydrophilic segment of the MBP signal peptide, whereas certain suppressor alleles of the *prlA* gene did not promote the export of the same MBP species.

Several studies have led to the proposal that the hydrophilic segment may also play a role in an obligatory coupling of the translation of exported proteins to their secretion. Inouye and coworkers (19, 41) found that reducing the net positive charge at the amino terminus of the lipoprotein (Lpp) signal peptide from +2 to −2 resulted in an accumulation of precursor Lpp, as well as a decrease in total Lpp synthesis. Hall et al. (13) observed that a double mutation (*lamB701-708*) that resulted in the removal of one of the two basic residues from the LamB signal peptide caused a decrease in LamB synthesis but did not appear to otherwise affect LamB export to the outer membrane. In contrast,

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other studies have cast some doubt on the existence of a mechanism in which the hydrophilic segment would be required to couple protein export to translation. Puziss et al. (25) observed that stepwise decreases in the net charge of the MBP signal peptide resulted in a progressive decrease in MBP synthesis, as well as a progressively severe export defect. However, when MBP export was completely blocked by the introduction of a hydrophobic core alteration in *cis* to a negatively charged hydrophilic segment, no increase in MBP expression was observed. In addition, the presence of the *prlD2* allele did not improve the expression of MBP species with alterations in the hydrophilic segment, even though it was capable of suppressing the export defect associated with such alterations. In both of these experiments, an increase in MBP expression would be expected if MBP synthesis and export were coupled. Finally, Iino et al. (16) observed that mutational alterations that decreased the net charge of the staphylokinase signal peptide did not result in decreased expression of staphylokinase when expressed in *E. coli*, although export was affected by these alterations. Thus, it remains unclear whether the basic residues of the hydrophilic segment play a role in coupling translation to protein export.

In this study, the role of the hydrophilic segment in the export and synthesis of MBP was investigated. The complete deletion of the hydrophilic segment resulted in a defect in the export of MBP, as well as a dramatic reduction in total MBP synthesis. Suppressor mutations that did not affect MBP export but that were shown to partially restore MBP synthesis at the level of translational initiation were isolated. These mutations were shown to lie upstream of the *malE* coding region. In other experiments, a large number of substitutions were introduced into the coding region, in the second codon of several *malE* alleles, and the effects of these substitutions on MBP export and synthesis were analyzed. In addition, the effects of alterations in the hydrophilic segment of the ribose-binding protein (RBP) signal peptide fused to the mature moiety of the MBP on hybrid protein export and synthesis were examined. Taken together, the data presented demonstrate that the hydrophilic segment of the MBP signal peptide is not involved in a mechanism that obligatorily couples protein export and translation. Rather, it is believed that mutations in this region of the *malE* gene affect translation by altering *malE* mRNA secondary structure, by altering a primary sequence that may be preferred for translational initiation, by introducing inefficiently translated codons into the *malE* mRNA, or by some as yet undescribed mechanism.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* BAR1091, a derivative of MC4100 (7), has been described previously (28). It harbors the *malEΔ312* mutation, an in-frame, nonpolar deletion that removes the DNA sequence encoding from residue 15 of the MBP signal peptide through residue 159 of the mature moiety. *E. coli* BL21(DE3) (39) harbors the bacteriophage T7 gene *1* (encoding T7 RNA polymerase) on a λ prophage and was kindly provided by F. W. Studier. The *lacUV5 malE* plasmids pJF2 and pUZ226 are described elsewhere (12, 46), as is the *lacUV5 rbsB-malE* plasmid pSMS41 (10).

Reagents. Minimal medium M63 supplemented with a carbon source (0.2%) and thiamine (2 μg/ml), maltose tetrazolium, and TYE agar were prepared as described previously (22). When required, ampicillin was added to minimal and complex media at concentrations of 25 and 50 μg/ml, respectively. To induce *malE* genes under *lacUV5* promoter-operator control (28), isopropyl-β-D-thiogalactopyranoside (IPTG) was used on agar plates and in liquid media at concentrations of 1 and 5 mM, respectively. [^{35}S]methionine (Expre^{35}s^{35}s) was obtained from New England Nuclear, Wilmington, Del. Rabbit anti-MBP serum has been described previously (11). Electrophoresis reagents were purchased from Life Technologies, Gaithersburg, Md. DNA-modifying enzymes were purchased from Life Technologies, Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Promega (Madison, Wis.). XAR film was purchased from Eastman Kodak Co., Rochester, N.Y.

Oligonucleotide-directed mutagenesis. Oligonucleotide-directed mutagenesis was employed to construct plasmids pUZ1 (encoding MBPΔ2-8), pUZ38 (encoding MBPΔ3-8), and derivatives of pUZ1, pUZ38, pJF2 (encoding wild-type MBP), pUZ226 (encoding MBPΔ2-26), and pSMS41 (encoding a hybrid protein with the RBP signal peptide fused to the MBP mature moiety). Mutagenesis was performed as previously described (26). When appropriate, mutagenic primers were designed to introduce any of the four possible nucleotides randomly at a single position. Oligonucleotides were prepared with an Applied Biosystems 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE) as described by Hutchison et al. (15). Plasmids packaged as M13 particles were prepared as previously described (40). Mutations were confirmed by DNA sequencing as described by Bankier et al. (3).

Radiolabeling, immunoprecipitation, SDS-PAGE, and autoradiography. Cultures were grown to the mid-log phase in minimal medium containing glycerol supplemented with ampicillin and induced for synthesis of MBP by the addition of IPTG to the culture medium. After 45 min, cells were labeled with [^{35}S]methionine for 15 s. Chase periods were initiated and terminated as described previously (31). For cellular fractionation experiments, a miniversion of the cold osmotic shock procedure of Neu and Heppel (23) was used as described previously (11). For quantitation of MBP synthesis, cultures at an identical optical density at 600 nm were radiolabeled for 10 min with [^{35}S]methionine. Radiolabeled MBP was immunoprecipitated from solubilized cell extracts by procedures described previously (31). Immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-PAGE and autoradiography, also as previously described (31). An AMBIS radioanalytic imaging system (AMBIS Systems, San Diego, Calif.) was employed to determine the radioactivity present in MBP bands in dried gels. Counts were adjusted for the loss of methionine residues when precursor MBP (pre-MBP) species were processed to mature MBP (mMBP).

In vitro analysis of translational initiation. Plasmids pBS-JF2B, pBS-UZ1B, and pBS-UZ112B were constructed as follows. First, plasmids pUZ1B, pJF2B, and pUZ112B were constructed by introducing a unique *Bam*HI site at a site corresponding to the first two codons of the mMBP coding sequence in plasmids pJF2 (*malE*$^+$), pUZ1 (*malEΔ2-8*), and pUZ112 (*malEΔ2-8sup1*) by oligonucleotide-directed mutagenesis. Introduction of the *Bam*HI site did not disrupt the *malE* reading frame, and MBP expression from the resulting plasmids, pJF2B, pUZ1B, and pUZ112B, was not affected (data not shown). An *Eco*RI-*Acc*I fragment harboring the entire *malE* gene was isolated from plasmids pJF2B, pUZ1B, and pUZ112B by electrophoresis through a low-melting-point agarose gel. DNA fragments were purified from gel slices by phenol extraction as previously described (34) and ligated into the multiple cloning site of plasmid

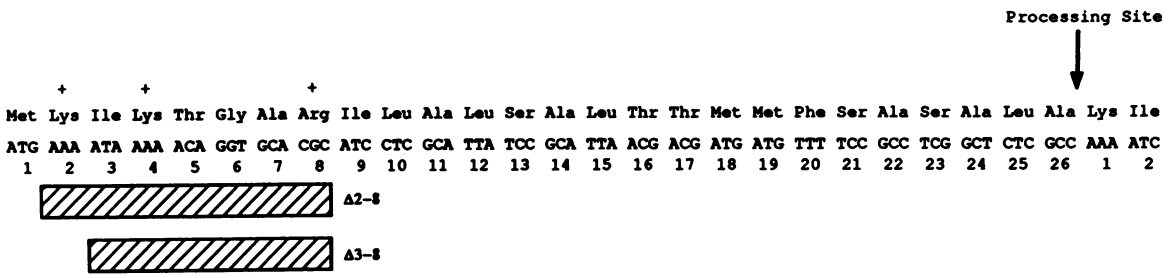

FIG. 1. Primary amino acid and nucleotide sequence of the MBP signal peptide. Hatched boxes indicate the sequences deleted by oligonucleotide-directed mutagenesis in order to construct MBPΔ2-8 and MBPΔ3-8.

pBS+ (Stratagene). The resulting plasmids were designated pBS-UZ1B, pBS-JF2B, and pBS-UZ112B. These constructions placed the various *malE* alleles under the control of the bacteriophage T7 promoter. In vitro transcription of the *malE* gene was carried out as described previously (32). In vitro translation and quantitation of the rate of synthesis of the 2-amino-acid initiation peptide Met-Ile from *malEΔ2-8* and *malEΔ2-8sup1* mRNAs was performed essentially as described by Weissbach and coworkers (9, 24, 30). The isolation of 70S initiation complexes was carried out as described previously (29).

RESULTS

Deletion of the hydrophilic segment of the MBP signal peptide. Plasmid pJF2 encodes the wild-type MBP under regulatory control of the *lacUV5* promoter-operator (12). Codons 2 through 8 of the *malE* gene, encoding the hydrophilic segment of the MBP signal peptide (Fig. 1), were precisely deleted from plasmid pJF2 by oligonucleotide-directed mutagenesis (see Materials and Methods). The plasmid harboring this *malEΔ2-8* deletion was designated pUZ1. Cells of strain BAR1091 harboring pUZ1 exhibited a strongly Mal$^-$ phenotype on maltose tetrazolium agar and were unable to grow on minimal agar plates containing maltose. However, after incubation for 2 days at 37°C, individual colonies representing Mal$^+$ pseudorevertants of BAR1091(pUZ1) were easily obtained.

Plasmid DNA from several independently isolated Mal$^+$ pseudorevertants was packaged into phage M13 coats by infection with M13KO7 helper phage (40). In each instance, transduction of packaged plasmid DNA into cells of strain BAR1091 demonstrated that the suppressor mutations conferring the Mal$^+$ phenotype were plasmid linked. DNA sequence analysis revealed that the suppressor mutations were located from 63 to 66 bases upstream of the *malE* coding sequence, just 3′ of the transcriptional start site of the *lacUV5* promoter. Four unique suppressor mutations were recognized: (i) a G-to-T transversion 9 bases downstream from the transcriptional start site (harbored on plasmid pUZ111), (ii) a G-to-A transition 12 bases downstream (on pUZ112), (iii) a G-to-A transition 9 bases downstream (on pUZ132), and (iv) a C-to-T transition 10 bases downstream (on pUZ162).

The finding that the suppressor mutations were located 5′ to the *malE* coding region suggested that MBP synthesis was affected by the *malEΔ2-8* deletion and the corresponding suppressor mutations. To determine the levels of MBP synthesis in these strains, cells harboring plasmid pUZ1 or its derivatives described above were radiolabeled for 10 min with [^{35}S]methionine. Radiolabeled MBP species were subsequently analyzed by immunoprecipitation, SDS-PAGE, and autoradiography (Fig. 2). As an internal standard for these experiments, OmpA protein was simultaneously immunoprecipitated from the solubilized cell extracts. The levels of synthesis of MBPΔ2-8 encoded by each of the mutant plasmids, relative to wild-type MBP encoded by pJF2, were quantitated as described in Materials and Methods. The deletion of the hydrophilic segment resulted in a dramatic reduction in the total amount of MBP synthesized, with MBPΔ2-8 being synthesized at a level only 14% that of wild-type MBP. Pulse-chase studies (data not shown) ruled out the possibility that the apparent decrease in synthesis was actually due to an increased rate of protein turnover. The suppressor mutations increased MBP synthesis to levels ranging between 28 (Fig. 2, lanes E and F) to 69% (lane D) that of wild-type MBP.

In addition to causing a decrease in MBP translation, the deletion of the hydrophilic segment of the signal peptide had a marked effect on MBP export. Several different forms of MBPΔ2-8 were precipitated, and the one migrating identically to wild-type MBP, a relatively minor band, was the only one released from cells by a cold osmotic shock (data not shown). The extremely small amount of mMBP produced by cells harboring plasmid pUZ1 accounts for their Mal$^-$ phenotype. The major protein bands seen in suppres-

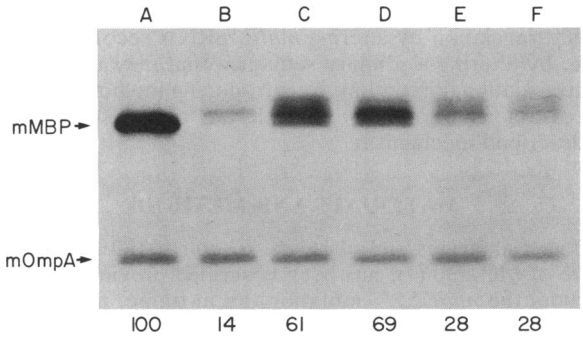

FIG. 2. Relative synthesis levels for wild-type MBP (lane A), MBPΔ2-8 (lane B), and pseudorevertants of MBPΔ2-8 encoded by plasmids pUZ112 (lane C), pUZ111 (lane D), pUZ132 (lane E), and pUZ162 (lane F) (see Results). Mid-log-phase cell cultures of strain BAR1091 at an identical optical density at 600 nm and harboring the appropriate plasmids were radiolabeled for 10 min with [^{35}S]methionine and solubilized. The MBP and OmpA (as an internal standard) were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. Quantitation was performed as described in Materials and Methods. Numbers at the bottom indicate relative levels of MBP synthesis, expressed as a percentage of wild-type MBP synthesis. mOmpA, mature OmpA.

TABLE 1. Formation of the 70S initiation complex with different mRNAs[a]

mRNA	mRNA in 70S complex	
	Amt (pmol/nmol of ribosomes)	% of *malE*$^+$ mRNA amt
malE$^+$	22.7	100
malEΔ2-8	0.82	3.6
malEΔ2-8sup1	4.8	21

[a] Wild-type *malE*, *malEΔ2-8*, and *malEΔ2-8sup1* (encoded by pUZ112) mRNAs were incubated in the presence of ribosomal subunits and initiation factors, and the relative amounts of mRNA present in 70S initiation complexes were determined as described in Materials and Methods.

TABLE 2. Rate of formation of the initiation peptide encoded by different mRNAs[a]

mRNA	Rate of synthesis of fMet-Ile	
	Rate (pmol/min/nmol of ribosomes)	Relative to *malEΔ2-8* mRNA
malEΔ2-8	0.069 ± 0.011	1.0
malEΔ2-8sup1	1.34 ± 0.13	17.7

[a] *malEΔ2-8* and *malEΔ2-8sup1* mRNAs were used to program a limited in vitro translation system, and the rate of synthesis of the 2-amino-acid initiation peptide fMet-Ile was determined as described in Materials and Methods.

sors of MBPΔ2-8 (Fig. 2, lanes C to F) migrated between the mature and intact precursor forms of MBPΔ2-8, and these proteins appeared to be stable, cytoplasmic breakdown products of pre-MBPΔ2-8. Similar intermediate forms have been previously observed for a number of export-defective MBP species with signal peptide alterations (2, 31).

In vitro analysis of translational initiation from *malEΔ2-8* and *malEΔ2-8* suppressor mRNAs. In light of the results described above, we hypothesized that the differences in the level of synthesis between wild-type MBP, MBPΔ2-8, and MBPΔ2-8 with upstream suppressor mutations might be due to differences in the efficiency of translational initiation. Therefore, it was decided to examine the initiation of translation from *malEΔ2-8* and *malEΔ2-8sup1* (encoded by pUZ112) mRNAs in vitro. To accomplish this, plasmids suitable for generating in vitro transcripts were constructed (see Materials and Methods). The resulting plasmids, pBS-JF2B, pBS-UZ1B, and pBS-UZ112B, directed the in vivo expression of MBP under the control of T7 RNA polymerase in cells of strain BL21 (39) at the same relative levels as from the *lacUV5* promoter (data not shown).

Purified DNAs from plasmids pBS-JF2B, pBS-UZ1B, and pBS-UZ112B were linearized by digestion with *Bam*HI. ^{32}P-labeled run-off transcripts initiated from the T7 promoter were prepared in vitro (32). Equal amounts of the mRNAs were then incubated in cocktails containing 30S and 50S ribosomal subunits and initiation factors, and the amount of 70S initiation complex formed was determined as previously described (29) (Table 1). It was found that *malEΔ2-8* mRNA supported the formation of 70S initiation complexes at a level only 3.6% that of wild-type *malE* mRNA. The presence of the upstream suppressor mutation in *malEΔ2-8sup1* mRNA increased the formation of the initiation complex to a level 21% that of the wild type.

A second in vitro assay was employed to measure the rate of translational initiation from *malEΔ2-8* and *malEΔ2-8sup1* mRNAs. A limited in vitro translation reaction in which only the first two tRNAs are present was performed, using *malEΔ2-8* and *malEΔ2-8sup1* mRNAs as substrates. The rate of synthesis of the 2-amino-acid initiation peptide fMet-Ile was then determined as previously described (9, 24, 30) (Table 2). Results from this analysis revealed that the rate of translation of the initiation peptide from *malEΔ2-8sup1* mRNA was approximately 18 times higher than from *malEΔ2-8* mRNA. Together, the results from the two in vitro assays demonstrated that the defect in MBPΔ2-8 synthesis is at the level of translational initiation and that the *malEΔ2-8sup1* mRNA was more efficient at initiating translation than *malEΔ2-8* mRNA was.

Effects of mutations at the second codon of *malEΔ3-8* on MBP export and synthesis. The deletion of the seven codons corresponding to the hydrophilic segment of the signal peptide significantly reduced total MBP synthesis. To further investigate any possible role of this structure in coupling MBP export and synthesis, a second in-frame deletion mutation in the *malE* gene (*malEΔ3-8*) was generated by oligonucleotide-directed mutagenesis (Fig. 1). This deletion removed DNA sequences encoding residues 3 through 8 from the MBP signal peptide, thus preserving the basic Lys residue at position 2. Cells harboring the resulting plasmid (designated pUZ38) synthesized MBPΔ3-8 at a level approximately 75% that of wild-type MBP (data not shown). Thus, the retention of the second codon (AAA) in the *malE* message and/or Lys as the second residue of the MBP signal peptide made a significant difference in the amount of MBPΔ3-8 synthesized compared with cells producing MBPΔ2-8 (75 versus 14%, respectively).

To further investigate the role of the second codon of *malE* in the translation and/or export of MBP, a variety of base substitutions were introduced within the second codon of the *malEΔ3-8* gene. MBP synthesis in cells harboring plasmid pUZ38 or various mutant derivatives was quantitated as previously described; the results of these experiments are shown in Fig. 3. Base changes within the second codon of *malEΔ3-8* affected total MBP synthesis to various degrees. The effects on synthesis could be quite pronounced, even in the case of conservative codon changes. Strikingly, the substitution of two different Arg-encoding codons resulted in MBPΔ3-8 derivatives synthesized at 91% (AGA) or only 9% (CGA) of the parental level. Note that the substitution of an Arg-encoding codon for a Lys-encoding codon in *malEΔ3-8* retains a net charge of +1 in the hydrophilic segment of the MBP signal peptide. Both AGA and CGA are considered rare codons in *E. coli* (6, 17), but the introduction of the CGA codon further downstream in the *malE* gene (codon 98 of the mMBP coding sequence) reduced MBP synthesis to 66% of the parental level (data not shown). In contrast, the substitution of a neutral Thr residue (ACA codon) for Lys at the same position had no apparent effect on MBPΔ3-8 synthesis. However, substitution of Thr for Lys did have an adverse effect on MBP export, as indicated by the increased accumulation of pre-MBP. The presence of other polar but uncharged residues at this position (e.g., Ser) also resulted in increased accumulation of pre-MBP. In general, it appeared that export of MBPΔ3-8 species was most efficient when the second residue was basic (Lys or Arg) or hydrophobic (e.g., Leu or Ile).

The export kinetics of MBPΔ3-8 and derivatives having either Arg (CGA) or Thr (ACA) at the second position were compared with that of wild-type MBP in a pulse-chase experiment (Fig. 4; see Materials and Methods). MBP processing was used as the indicator of export to the periplasm

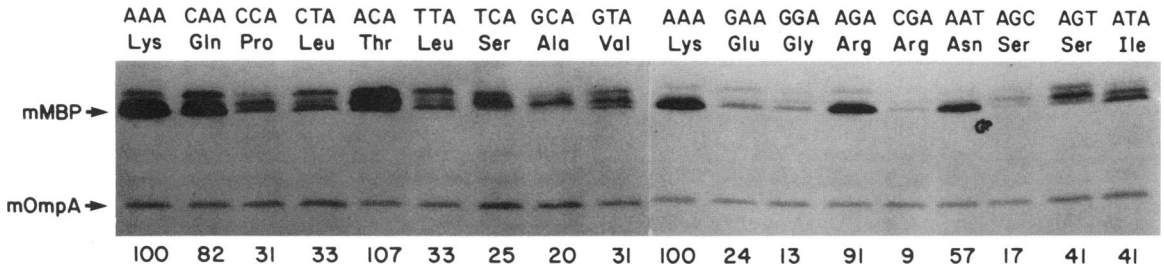

FIG. 3. Relative synthesis levels for MBPΔ3-8 (AAA Lys lanes) and derivatives with alterations in the second residue of the signal peptide. Cells of strain BAR1091 harboring plasmid pUZ38 or its derivatives were radiolabeled, and MBP and OmpA were immunoprecipitated and analyzed as described in the legend to Fig. 2. The nucleotide sequence of the second codon of the *malE* allele and the amino acid encoded by it are indicated above each lane. Numbers at the bottom indicate relative levels of MBP synthesis, expressed as a percentage of MBPΔ3-8 synthesis. mOmpA, mature OmpA.

(2, 11, 31). As expected, wild-type MBP was exported rapidly, with very little pre-MBP discernible by the 1-min chase point. MBPΔ3-8 was exported with somewhat slower kinetics, with approximately 50% maturation by the 1-min chase point, but exhibited virtually 100% maturation after 10 min of chase. Despite being synthesized at a much lower level, MBPΔ3-8(Arg[CGA]@2) was exported with kinetics that were nearly identical to those of the parental MBPΔ3-8. In striking contrast, MBPΔ3-8(Thr[ACA]@2) was synthesized at a level equivalent to that of MBPΔ3-8 but was exported with significantly slower kinetics, with only a small amount of authentic mMBP discernible at the 10-min chase point. (As encountered previously, the majority of the nonexported precursor form of this MBP species was partially degraded to a smaller, intermediate form by the 10-min chase point.)

Effects of mutations at the second codon of wild-type MBP on translation of MBP. In previous experiments with MBP species harboring deletions of the hydrophilic segment, the basic residues normally found at positions 4 and 8 of the MBP signal peptide were absent. Therefore, it was of interest to examine the effects of mutations in the second codon of an otherwise wild-type MBP species. To this end, oligonucleotide-directed mutagenesis was employed to substitute the first 2 bp of the second codon of wild-type *malE* with any of the four nucleotides at random (see Materials and Methods). MBP synthesis was quantitated for cells harboring these mutant derivatives of pJF2 as described above (Fig. 5). None of the mutations appeared to affect MBP export kinetics, as evidenced by the absence of accumulation of pre-MBP. This was not surprising, as all of these altered MBP species still retained two basic residues, at positions 4 and 8 of the signal peptide. However, nucleotide changes in the second codon of an otherwise wild-type *malE* gene could have marked effects on the level of MBP synthesis, in the absence of any effect on MBP export. For example, an ATA (Ile) codon introduced at this position reduced MBP synthesis to a level only 32% that of wild-type MBP. It was previously demonstrated that the substitution of an ATA encoding Ile for AAA encoding Lys at codon 4 of the hydrophilic segment of an otherwise wild-type MBP signal peptide does not significantly affect either the amount of MBP synthesis or MBP export kinetics (25). In addition, a CGA (Arg) codon introduced at this position decreased MBP synthesis to only 17% of that obtained with the wild-type gene, despite the fact that this MBP species retained a net charge of +3 within the hydrophilic segment and exhibited no detectable export defect.

Effects of mutations in the second codon of *malEΔ2-26* on MBP synthesis. In order to investigate the role in translation of the second codon of the *malE* gene in a context that was completely independent of secretion, it was decided to introduce a variety of mutations into the second codon of a totally export-incompetent MBP species, MBPΔ2-26. Plasmid pUZ226 harbors the *malEΔ2-26* allele, which encodes MBPΔ2-26, an MBP species from which the entire signal peptide (residues 2 through 26) has been deleted (46). Not surprisingly, MBPΔ2-26 is totally export incompetent and probably does not engage the secretion apparatus.

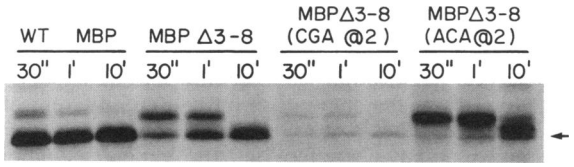

FIG. 4. Comparison of export kinetics for wild-type MBP (WT MBP), MBPΔ3-8, and derivatives of MBPΔ3-8 with either Arg (encoded by CGA) or Thr (encoded by ACA) at the second residue of the MBP signal peptide. Mid-log-phase cell cultures of strain BAR1091 at an identical optical density at 600 nm and harboring the appropriate plasmids were pulse-radiolabeled with [^{35}S]methionine for 15 s and chased with excess unlabeled methionine. At the time points indicated at top of the gel (30 s [30"], 1 min [1'], or 10 min [10']), equal portions were removed, the chase was terminated, and the MBP was immunoprecipitated and analyzed as described in the legend to Fig. 2. The position of mMBP is indicated by the arrow to the right of the gel.

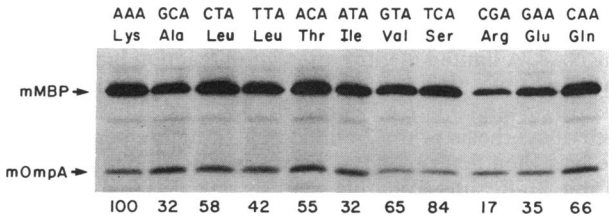

FIG. 5. Relative synthesis levels for wild-type MBP (AAA Lys lane) and derivatives with alterations at the second residue of the signal peptide. Cells of strain BAR1091 harboring plasmid pJF2 or its derivatives were radiolabeled, and MBP and OmpA were immunoprecipitated and analyzed as described in the legend to Fig. 2. The nucleotide sequence of the second codon of the *malE* allele and the amino acid encoded by it are indicated above each lane. Numbers at the bottom indicate relative levels of MBP synthesis, expressed as a percentage of wild-type MBP synthesis. mOmpA, mature OmpA.

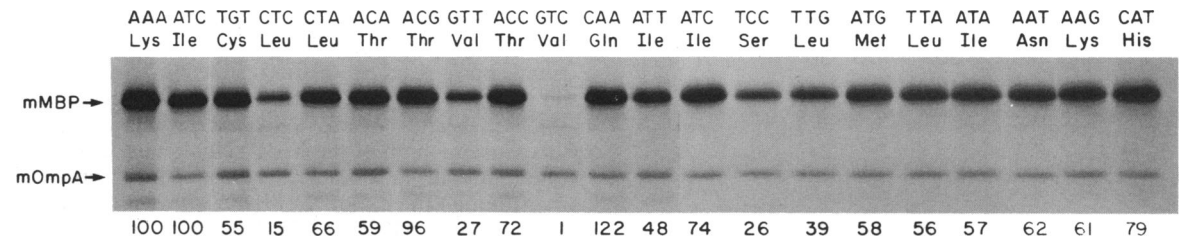

FIG. 6. Relative synthesis levels for MBPΔ2-26 (AAA Lys lane) and derivatives with alterations at the second residue of MBPΔ2-26 (i.e., the first residue of mMBP). Cells of strain BAR1091 harboring plasmid pUZ226 or its derivatives were radiolabeled, and MBP and OmpA were immunoprecipitated and analyzed as described in the legend to Fig. 2. The nucleotide sequence of the second codon of the *malE* allele and the amino acid encoded by it are indicated above each lane. Numbers at the bottom indicate relative levels of MBP synthesis, expressed as a percentage of MBPΔ2-26 synthesis. mOmpA, mature OmpA.

MBPΔ2-26 is synthesized at levels slightly greater than that of wild-type MBP (data not shown). Oligonucleotide-directed mutagenesis was employed to substitute any of the four nucleotides randomly at all three positions within the second codon of *malEΔ2-26*, which is normally AAA encoding Lys. MBP synthesis in cells harboring these mutant plasmids was quantitated as described above (Fig. 6). As observed for mutations in the second codon of wild-type *malE* and *malEΔ3-8*, changes in the second codon of *malEΔ2-26* that had a significant effect on total MBPΔ2-26 synthesis, even in the case of conservative codon changes, were identified. For example, MBPΔ2-26 with a CTC-encoded Leu at position 2 was synthesized at a level 15% that of MBPΔ2-26, whereas MBPΔ2-26 with a CTA-encoded Leu at position 2 was synthesized at a level 66% that of MBPΔ2-26. Likewise, MBPΔ2-26 with a GTT-encoded Val at position 2 was synthesized at a level 27% that of wild-type MBP, whereas MBPΔ2-26 with a GTC-encoded Val at position 2 was synthesized at a level only 1% that of MBPΔ2-26. Thus, it was clear that mutations in the second codon of *malEΔ2-26* could have dramatic effects on the synthesis of MBPΔ2-26. Note that the MBPΔ2-26 species which exhibited the highest relative synthesis level harbored a neutral amino acid, Gln encoded by CAA.

Mutational analysis of the hydrophilic segment of an RBP-MBP hybrid protein. Plasmid pSMS41 encodes a hybrid protein that consists of the signal peptide of the RBP fused to the mature moiety of MBP. The RBP signal peptide harbors two basic Lys residues in the hydrophilic segment (both encoded by AAA) at residues 4 and 5 (Fig. 7). Oligonucleotide-directed mutagenesis was employed to substitute the two lysines in the hydrophilic segment with two hydrophobic residues, Ile at residue 4 encoded by ATA and Leu at residue 5 encoded by TTA. The resulting plasmid, pUZ11, directed hybrid protein expression at a level 12.9% that of the parental plasmid pSMS41 (data not shown). Beginning with plasmid pUZ11 as a substrate, oligonucleotide-directed mutagenesis was used to insert the sequence CGCCGC, encoding two Arg residues, at codons 6 and 7. The Arg codon CGC is commonly utilized by *E. coli* (17). The resulting plasmid, pUZ12, encoded a hybrid protein that possessed a net positive charge of +2 within the hydrophilic segment and directed expression of the hybrid protein at a level 6.8% that directed by pSMS41 (data not shown).

The export kinetics of the hybrid proteins encoded by pSMS41, pUZ11, and pUZ12 were examined by pulse-chase analysis (Fig. 8). The hybrid protein encoded by pSMS41 is exported rapidly, with only a small amount of precursor protein relative to mature protein discernible at the earliest chase point. In contrast, the hybrid protein encoded by

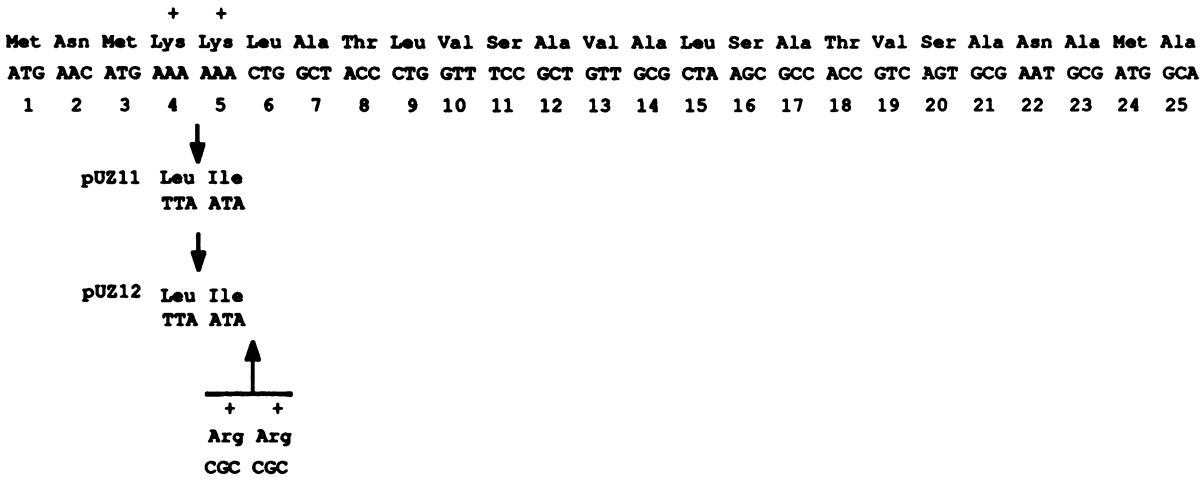

FIG. 7. Primary amino acid and nucleotide sequence of the RBP signal peptide and derivatives with alterations in the hydrophilic segment. Plasmid pSMS41, encoding a hybrid protein consisting of the wild-type RBP signal peptide fused to the mature moiety of MBP, was subjected to oligonucleotide-directed mutagenesis (as described in Materials and Methods) in order to construct plasmids pUZ11 and pUZ12. Arrows indicate changes in primary amino acid sequence from the preceding construct. The upward-pointing arrow indicates an insertion of two Arg-encoding codons in pUZ12.

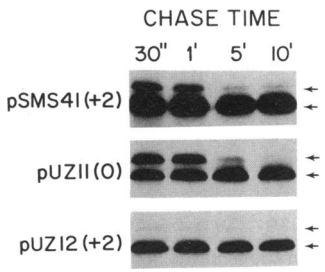

FIG. 8. Comparison of export kinetics for RBP-MBP hybrid proteins with a wild-type hydrophilic segment (encoded by plasmid pSMS41) or with altered hydrophilic segments (encoded by plasmids pUZ11 and pUZ12). Cells of strain BAR1091 harboring the appropriate plasmids were pulse-radiolabeled and chased with excess unlabeled methionine for various times, and the hybrid protein was immunoprecipitated and analyzed as described in the legend to Fig. 4. The chase times are indicated at top. The net charge of the hydrophilic segment of each hybrid protein species is indicated in parentheses after the plasmid designation. The positions of the precursor and mature forms of the hybrid proteins are indicated by the arrows to the right of the gel.

pUZ11 exhibited a marked export defect, with approximately 50% of the hybrid protein detected as precursor by the 1-min chase point. The hybrid protein encoded by pUZ12 exhibited very rapid export kinetics, with almost no precursor protein discernible at the earliest chase point. However, as noted above, the hybrid protein encoded by pUZ12 is synthesized at a level even lower than that encoded by pUZ11. Thus, the insertion of two Arg residues in the hydrophilic segment of the RBP signal peptide resulted in a marked improvement in export kinetics, while it did not increase levels of hybrid protein synthesis.

DISCUSSION

In an earlier study (25), the three basic residues at the amino terminus of the MBP signal peptide were systematically substituted with neutral or acidic residues, decreasing the net charge of this region in a stepwise fashion from +3 to −3. Export was most rapid and efficient when the signal peptide retained at least a single basic residue and a net charge of +1. When the net charge was decreased below +1, both the rate and efficiency of MBP export progressively decreased. However, even a signal peptide with a net charge of −3 could still facilitate the export of approximately 60% of the total MBP synthesized, as long as the hydrophobic core remained unaltered.

In this study, the deletion of residues from the hydrophilic segment was found to have a significantly stronger effect on MBP export than might have been predicted from the earlier results. MBPΔ2-8, with the entire hydrophilic segment deleted, exhibited a severe export defect; approximately 20% of the total MBP synthesized was exported to the periplasm. MBPΔ3-8, with the entire hydrophilic region deleted except for the Lys at residue 2, exhibited significantly slower export kinetics than wild-type MBP, despite the fact that it retained a net charge of +1 at its amino terminus. Still, essentially all of the MBPΔ3-8 synthesized was eventually exported and processed to mMBP. These results indicate that the presence of a single basic residue at the amino terminus of a truncated MBP signal peptide is not sufficient to facilitate MBP export at the rate of the wild type. It is not evident why this is the case. Although there are a number of prokaryotic signal peptides that contain only a single basic residue in their hydrophilic segment, there is no example in which a single basic residue constitutes the entire region (save for the initiating Met) (35). The reductions in the efficiencies of MBPΔ3-8 and MBPΔ2-8 export probably result from the decreased length of the MBP signal peptide, as well as from the reduction in the net charge of the hydrophilic segment.

Although the export of MBPΔ3-8 was not as rapid as that of wild-type MBP, it did provide a system for assessing individual amino acids for their ability to promote export when substituted at the second position of this truncated signal peptide. MBPΔ3-8 export was not affected by the substitution of Arg for Lys at position 2. Both of these basic residues are frequently encountered in the hydrophilic segments of signal peptides (35). Interestingly, the substitution of either Asn or Gln, both neutral residues, for Lys had only a minor adverse effect on MBPΔ3-8 export (Fig. 3), suggesting that the charge character of their carboxamide side groups can actively promote MBP export. Again, both Asn and Gln are commonly found in the hydrophilic segments of prokaryotic signal peptides (35). Note that Asn is found at position 2 of the RBP signal peptide (Fig. 7).

When the Lys at residue 2 of MBPΔ3-8 was converted to neutral residues other than Asn or Gln, a significant decrease in MBP export efficiency usually resulted, although in most of these instances export was somewhat more efficient than that exhibited by MBPΔ2-8. This again suggests that the length of the signal peptide may be important for optimal export efficiency. The substitution of a hydrophobic residue (e.g., Leu or Ile) at position 2 of MBPΔ3-8 resulted in somewhat more efficient export than the substitution of a polar but uncharged residue (e.g., Ser or Thr). This finding most likely results from the functional lengthening of the hydrophobic core by one residue. It was previously noted that lengthening the hydrophobic core of the MBP signal peptide could partially compensate for adverse changes in the hydrophilic segment and vice versa (25).

Derivatives of wild-type MBP with substitutions for the Lys at position 2 of the signal peptide displayed export kinetics that were indistinguishable from that of wild-type MBP. This result is not surprising, given that in each of these cases, the MBP signal peptide still retains two basic residues at positions 4 and 8 of the signal peptide. Thus, in the case of an MBP signal peptide with a net charge of +2, the position of the basic residues within the hydrophilic segment does not appear to be critical.

In addition to the observed defect in MBP export, the deletion of the hydrophilic segment in MBPΔ2-8 resulted in a striking reduction in overall MBP synthesis. Previous analyses of the hydrophilic segments of the LamB (13) and Lpp (19, 41) signal peptides have also yielded mutations which resulted in reduced expression of these proteins. However, to our knowledge the observed decrease in MBP synthesis caused by deletion of the hydrophilic segment is greater than any previously reported for other prokaryotic exported proteins. This vast reduction in MBP synthesis allowed for the selection of Mal^+ pseudorevertants of MBPΔ2-8. Interestingly, the only suppressors that arose harbored mutations 63 to 66 bases upstream of the *malEΔ2-8* coding region. These mutations could suppress the synthesis defect caused by deletion of the hydrophilic segment, but not the export defect caused by the same deletion. Examination of predicted *lacUV5 malEΔ2-8* mRNA secondary structure revealed a potential hairpin formed by base pairing between nucleotides 12 to 19 and nucleotides −59 through −66 relative to the initiation codon (data not shown). This

secondary structure could potentially interfere with ribosomal access to the initiation codon and/or Shine-Dalgarno sequence. The suppressor mutations have the potential to destabilize this structure, thereby allowing for more efficient translational initiation.

It has been proposed (13, 19) that mutations within the hydrophilic segment that decrease translation of exported proteins may do so through the operation of a putative coupling mechanism. Presumably, such a mechanism would cause an arrest in the elongation phase of translation in a manner similar to that imposed in vitro by the signal recognition particle in eukaryotes (45). In such a model, the basic residues of the hydrophilic segment would be necessary to release the imposed translational block. However, in the case of *malEΔ2-8*, it has been demonstrated in vitro that the defect in translation is not at the level of elongation, but at the level of translational initiation. A defect in the initiation of translation of an exported protein would, by definition, occur before any putative coupling mechanism could be engaged. Thus, the reduction in MBPΔ2-8 synthesis could not be due to the operation of a coupling mechanism.

In order to determine whether retention of a single basic residue in the hydrophilic segment was important for efficient MBP translation, MBPΔ3-8 and its derivatives were constructed. However, the levels of MBPΔ3-8 synthesis did not correlate with the presence or absence of a basic residue in the hydrophilic segment. For example, note that the MBPΔ3-8 species with CGA-encoded Arg was synthesized at a level only 9% that of the MBPΔ3-8 parent, despite the fact that this MBP species retains a net charge of +1 in the hydrophilic segment. In contrast, the MBPΔ3-8 species with the ACA-encoded Thr was synthesized at a level virtually indistinguishable from that of the MBPΔ3-8 parent. These results strongly suggest that there is no requirement for a basic residue in the hydrophilic segment for high-level MBP synthesis.

MBP species with alterations at the second residue in an otherwise wild-type signal peptide also displayed significant variations in the level of overall MBP synthesis, despite the fact that all of these MBP derivatives are exported with wild-type kinetics. These results show that mutations near the initiation codon in an otherwise wild-type *malE* gene can dramatically influence translation without affecting export. As was observed for MBPΔ3-8, the substitution of a CGA (Arg) codon for the AAA (Lys) codon at codon 2 resulted in the strongest decrease in MBP synthesis. It has been suggested that the CGA codon may be translated less efficiently than other codons because it is inefficiently recognized by its cognate tRNA (17). However, when this codon was placed at codon 98 of mMBP, the reduction in MBP synthesis was much less severe (data not shown). Thus, it appears that this codon exerts a stronger inhibitory effect on the rate of translation when it is placed in proximity to the initiation codon. It may be that the process of translational initiation is more sensitive to inefficiently translated codons than is the elongation phase of translation.

The effects of alterations at the second residue of an unexported, cytoplasmic MBP species were also examined. MBPΔ2-26 (46) is a totally export-incompetent MBP species that lacks a signal peptide, and therefore, it would be unlikely to engage any machinery that would normally couple export to translation. Interestingly, MBPΔ2-26 is synthesized at a higher level than is wild-type MBP expressed from a similar plasmid (data not shown). Mutations within the second codon of *malEΔ2-26* (the first codon of the mMBP, AAA encoding Lys) could have significant effects on the translation of MBPΔ2-26. For example, note the strong detrimental effect of the CTC, GTT, GTC, and TCC codons on MBPΔ2-26 translation. These results show that mutations near the initiation codon can exert an effect on MBP synthesis in the total absence of MBP export and, therefore, in the absence of any effect exerted by a putative coupling mechanism.

Experiments performed with RBP-MBP hybrid proteins also provided strong evidence against the existence of an obligate coupling of translation to protein export. As discussed above, substitution of the two Lys residues in the RBP signal peptide with two hydrophobic residues resulted in a decrease in translation of the hybrid protein, as well as a kinetic delay in export (Fig. 7). Insertion of two CGC codons encoding Arg at residues 6 and 7 restored the rate of export of the hybrid protein to wild-type levels. However, the insertion of the two Arg residues did not result in any increase in synthesis of the hybrid protein. Thus, it was clear that while insertion of the two Arg residues completely restored a functional hydrophilic segment in terms of protein export, it did not restore any function in terms of translation.

There are several possible explanations that could account for the effects of mutations in the hydrophilic segment on MBP translation, in lieu of a coupling model. For example, it is possible that mutations within *malE* sequences encoding the hydrophilic segment could influence the efficiency of translational initiation by altering mRNA secondary structure near the initiation codon or Shine-Dalgarno sequence. This seems to be the case for *malEΔ2-8*, as suggested by the isolation of suppressor mutations upstream of the coding region of *malEΔ2-8*. Looman et al. (21) have suggested that there may be a preference for A-rich codons immediately 3′ to the initiation codon on the basis of a mutational analysis of the second codon of *lacZ*. In addition, a preference for A-rich sequences immediately 3′ of the initiation codon has been suggested on the basis of statistical analysis (38). Adenine-rich sequences would be less likely to form base pairs and form regions of mRNA secondary structure. Conversely, C- and G-rich sequences were suggested to be detrimental to translational initiation, as they would possess greater potential to form mRNA secondary structures. It is interesting to note that the AAA (Lys) codon is the most common second codon in *E. coli* genes (38).

A number of studies have suggested that certain nucleotide sequences may be preferred within a 10- to 20-nucleotide region flanking the initiation codon (33, 38). It has been suggested that these sequences might influence mRNA selection by the ribosome. Mutations within the *malE* sequence encoding the hydrophilic segment could inhibit translational initiation by disrupting sequences that are preferred for translational initiation. Alternatively, it is possible that this preferred sequence reflects the potential for certain sequences to remain in a state that is relatively free of mRNA secondary structure.

It is difficult to draw conclusions based upon our data as to which of these mechanisms are responsible for the effects of mutations in the hydrophilic segment on MBP synthesis. However, it seems clear that mutations 3′ of the initiation codon in *malE* could influence translation of MBP at the level of initiation. This has been experimentally verified for MBPΔ2-8. Thus, we believe that the observed decreases in MBP synthesis are due to a mechanism other than one that would obligatorily couple protein export to translation. Iino et al. (16) have observed that mutations which decrease the net charge of the staphylokinase signal peptide do not result in a decrease in protein synthesis. These results, in addition

to those reported here, indicate that while the hydrophilic segment is important for rapid and efficient protein export, it does not play a role in obligatorily coupling protein export to translation. Several mutations in the hydrophilic segment of exported proteins in *E. coli* that cause a reduction in protein synthesis have been reported (13, 19). It is possible that these could be accounted for by one of the mechanisms discussed above, without invoking a coupling model. For example, it is possible that mutations that decrease the net charge of the hydrophilic segment of the LamB and Lpp signal peptides could subtly alter mRNA secondary structure near the translation initiation site. Such changes might not be detectable by currently available computer models or by visual inspection for potential secondary structures. In addition, these mutations could alter primary sequences that have been suggested to play a role in translational initiation.

It is possible that protein export and translation are coupled in some manner that does not involve the basic residues of the hydrophilic segment in *E. coli*. There are several lines of evidence that would suggest this. (i) Many proteins can be exported cotranslationally in *E. coli* (36, 37). (ii) It has been demonstrated that MBP with a wild-type signal peptide is synthesized by membrane-bound polysomes (27). (iii) Overproduction of the outer membrane protein OmpC (8) or the periplasmic protein GlpQ (14) may result in the reduction of synthesis of other outer membrane or periplasmic proteins, respectively. However, the results reported here indicate that care must be taken in interpreting mutational analyses of exported proteins in which mutations near the initiation site are observed to have an effect on translation. These mutations could exert an effect on protein synthesis because of a mechanism other than one which involves coupling of protein synthesis to protein export.

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